Evaluation of Novel Platforms to Differentiate Pathovars of Plant Pathogenic Bacteria

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The plant biosecurity issue

- Many of the biggest threats to Australia's plant industries are bacterial
- Identification to the subspecific ("pathovar") level is difficult
  - by definition, "pathovar" identifies host, but often the host range is incompletely defined
  - bioassays are slow, subjective, need quarantine facilities
  - rapid and robust tests not always available
    - serology, molecular, Fatty Acid Methyl Ester analysis
- However, decisions regarding response, management and/or market access require definitive identification to pathovar level

There are many pathovars but few robust protocols
Traditionally the process to develop such tests is slow and expensive

Objectives

- Evaluate the ability of new platforms to fast-track the identification of better biomarkers at pathovar level
  - Analysis of functional molecules - proteins and metabolites – between selected pathovars
  - Identify those that are differentially expressed
  - These may be associated with, or determinants of, pathogenicity (and therefore a stable identifier of the pathogen)

- Design better diagnostic assays
  - Use of whole genome sequences (international database) to identify genes = DNA-based diagnostics
  - Specific metabolites

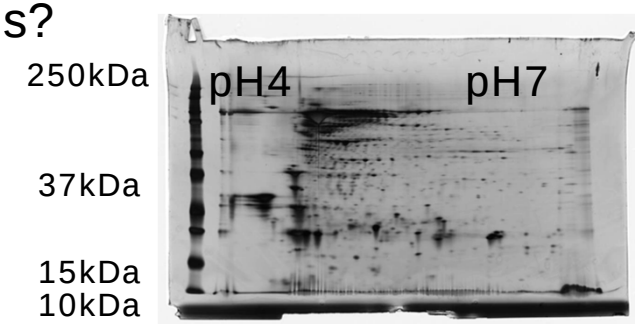
Proteomics

- Analysis of the proteins being expressed by an organism.

- Not used for plants but (eg)

  - Cordwell *et al*, 2008 *Proteomics* 8:122-139
    Identification of membrane-associated proteins in *Campylobacter*

Potential for development of (eg) hand-held tools?



- Methodology
  - Hydrophobic membrane fraction
  - Silver-stained 2D gels
  - Pattern recognition software (Non-Linear SameSpots)
  - Spots eluted and digested
  - Voyager MS and sequencing
  - Database (Mascot)

Model : *Xanthomonas*

- Major pathogens of wide range of economically important plant species
- Continual taxonomic revision
  - > 30 species and hundreds of pathovars
- Several whole genome sequences available

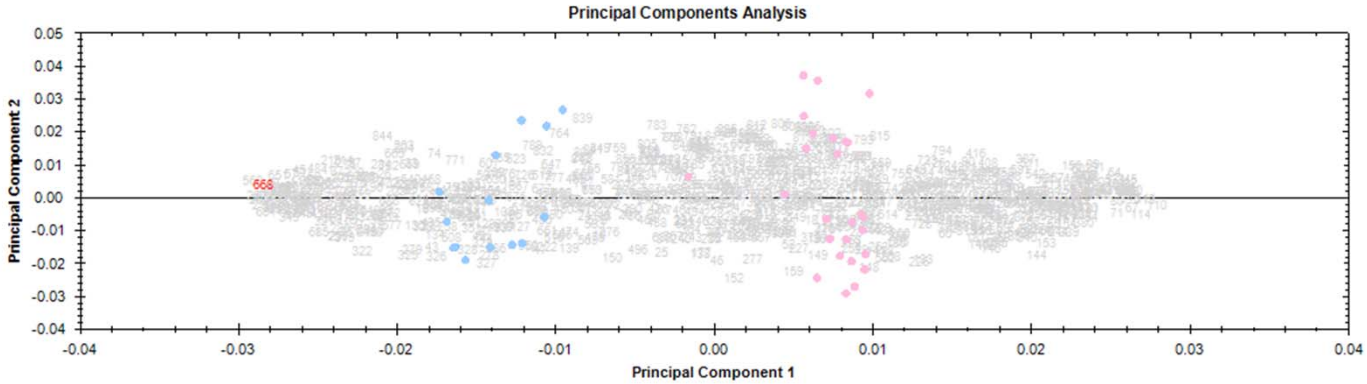
Species to use as the model?

- *X. axonopodis pv citri*
  - A vs A* vs Aw
  - A vs pv *malvacearum*
  - A vs E
- Multiple isolates of each

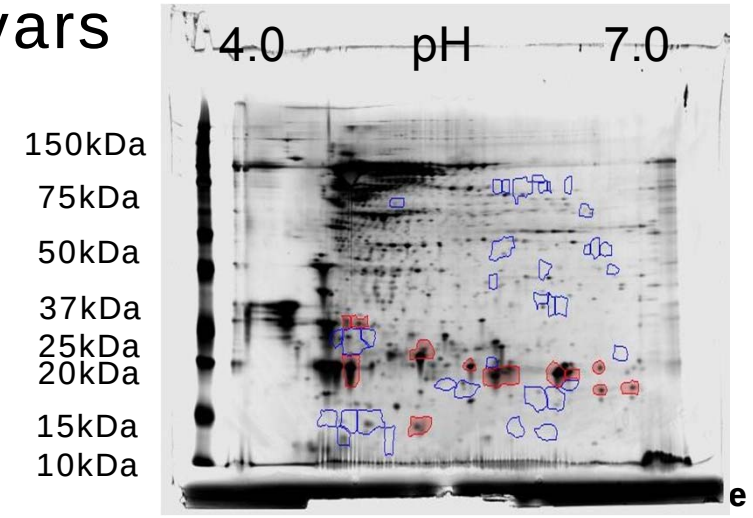
- Methodology - Membrane-bound (surface-mounted) proteins
  - potential role in host/pathogen interaction?

Proteomics - results

- Pathovars can be grouped according to expressed proteins



- Some proteins are differentially expressed between closely-related pathovars
  - Locate within genome
  - Diagnostic based on
    - Presence/absence
    - Deletions/insertions
    - SNPS



CRC PLANT biosecurity

Spot No.	Putative ID	Function?	No. of Primers Sets
1	Outer membrane protein	Unknown	2
4	Putative uncharacterised protein	Unknown	2
5	Outer membrane protein	Unknown	2
6	Outer membrane protein P6	Unknown	2
7	Outer membrane protein W	Higher levels of expression in oxidative stress (Asakura et al, 2008); modulation of expression in cells grown under stress such as elevated temps., high salt and low aeration (Nandi et al., 2005)	2
8	Outer membrane protein W		3
9	Outer membrane protein	unknown	2
14	Alkyl hydroperoxide reductase subunit C	Catalytic subunit responsible for alkyl peroxide metabolism (Mongkolsuk et al, 2000)	3
15	Polyphosphate-selective porin O	Small ion channel, including under conditions of overnight phosphate starvation (Hancock et al., 1992)	4
16	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MECDP)	An enzyme in the mevalonate-independent isoprenoid biosynthetic pathway (Richard et al., 2002). Catalyses the conversion of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate to MECDP. (what?)	2

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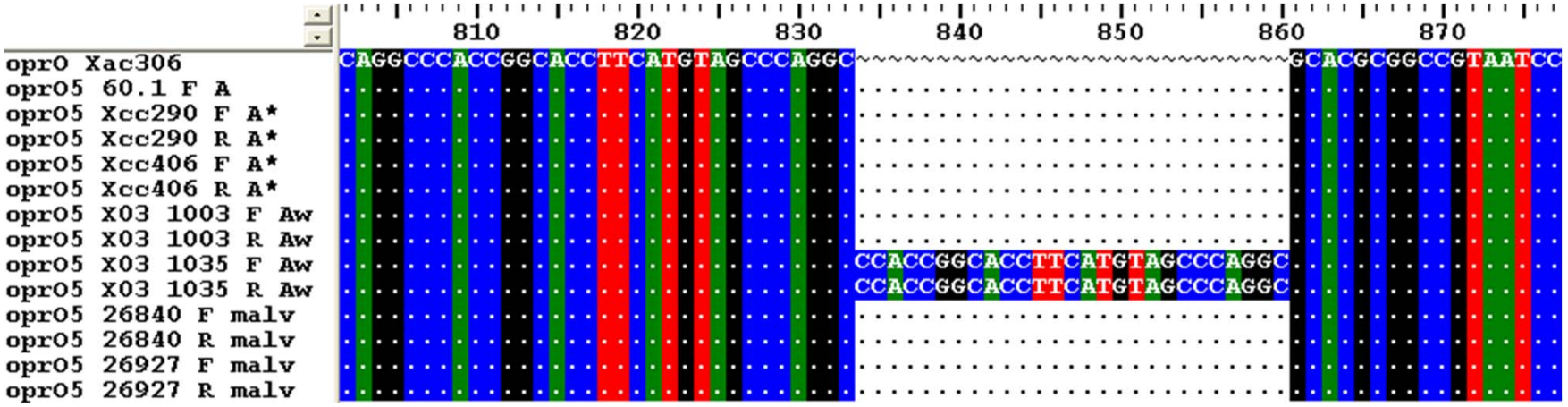
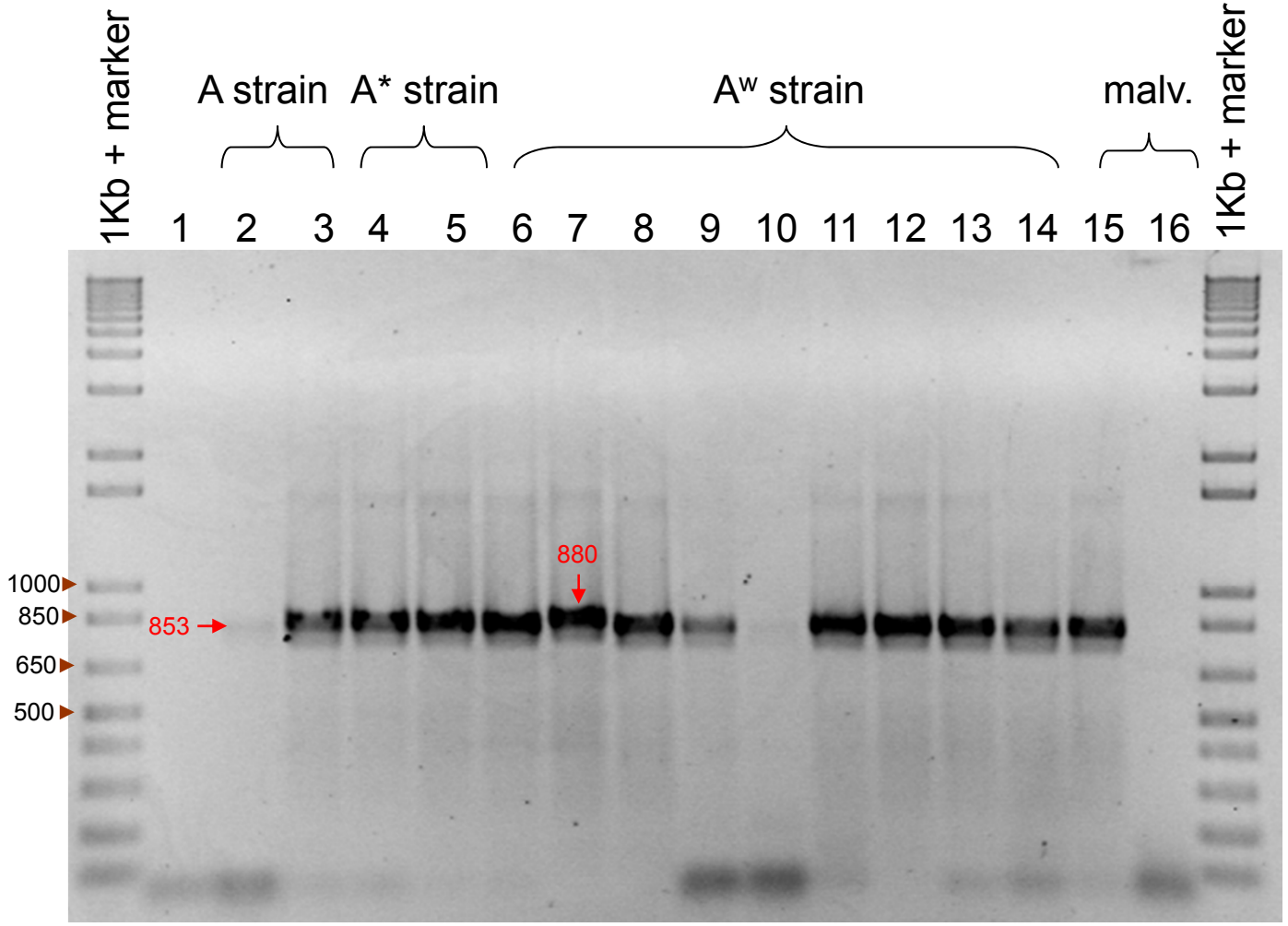
Nucleotide sequence analysis

- Aligned sequences shows 27bp insert in A^w isolate (X03 1035)



- Is this a valid difference between strains?



Lanes

1. Negative control
2. 60.1 □
3. 394.2 □
4. Xcc290 ▲
5. Xcc406 ▲
6. X03_1003 ○
7. X03_1035 ○
8. X2000-12884 ○
9. X2001-00005 ○
10. X2001-00032 ○
11. X2003-01008 ○
12. X2003-01012 ○
13. X2003-01029 ○
14. DAR26840 ◊
15. DAR26927 ◊
16. Negative control

CRC PLANT biosecurity

biosecurity **built on science**

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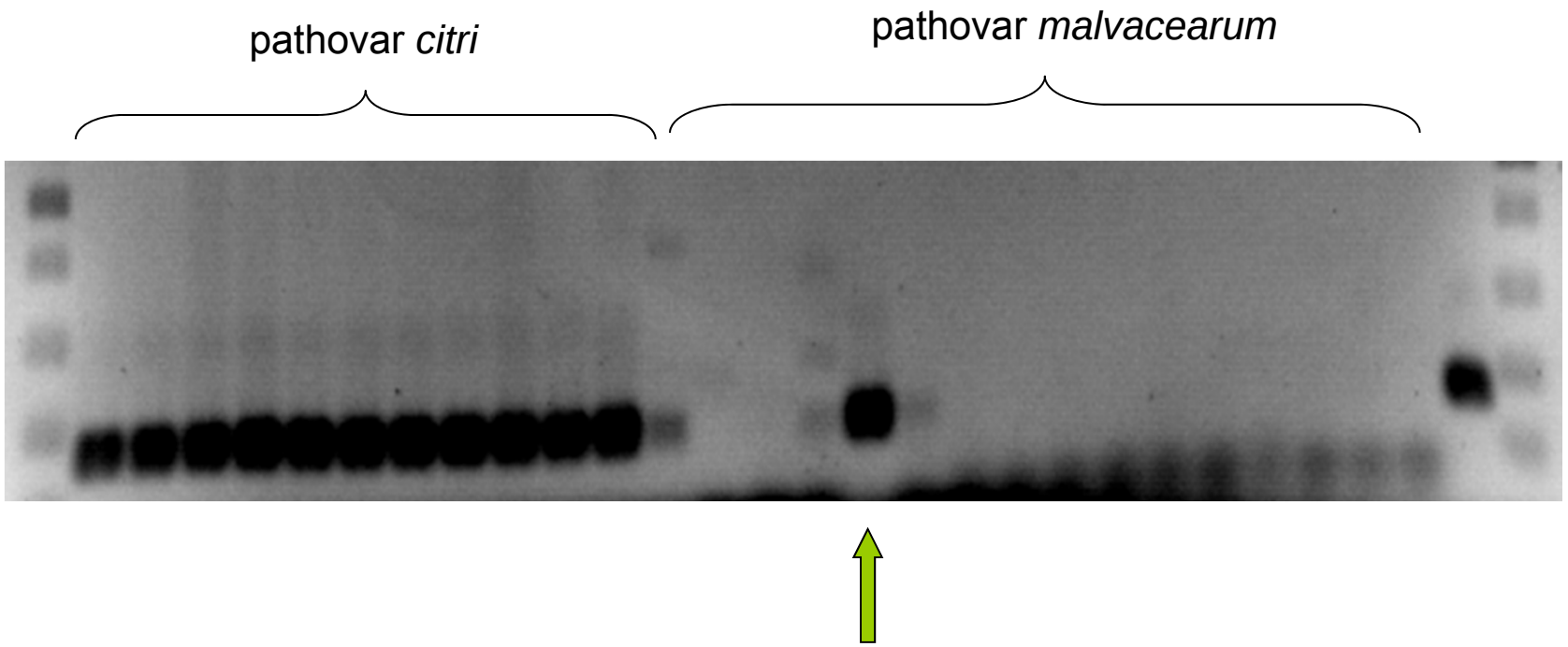
OMP W – a potential biomarker?



Next steps - proteomics

- Continue to validate identified biomarkers against collection of reference isolates for use as diagnostic

- Whole genome sequencing
  - to identify proteins not expressed in citri but detected in other pathovars
    - 6 Mbp only!

Metabolomics

- Metabolomics uses a combination of analytical techniques—mainly mass spectroscopy and nuclear magnetic resonance (NMR) spectroscopy

- Identify the compounds and their quantities, coupled with mathematical or statistical modelling to identify significant peaks and troughs.

- Examples
  - -Detecting metabolic changes eg. prostate and cervical cancer
  - -*H. pylori* detection in breath tests (volatile metabolomics)
  - -Meningococcus in CSF
  - -Pneumococci using urinary metabolomics

Model bacteria – *X. campestris*

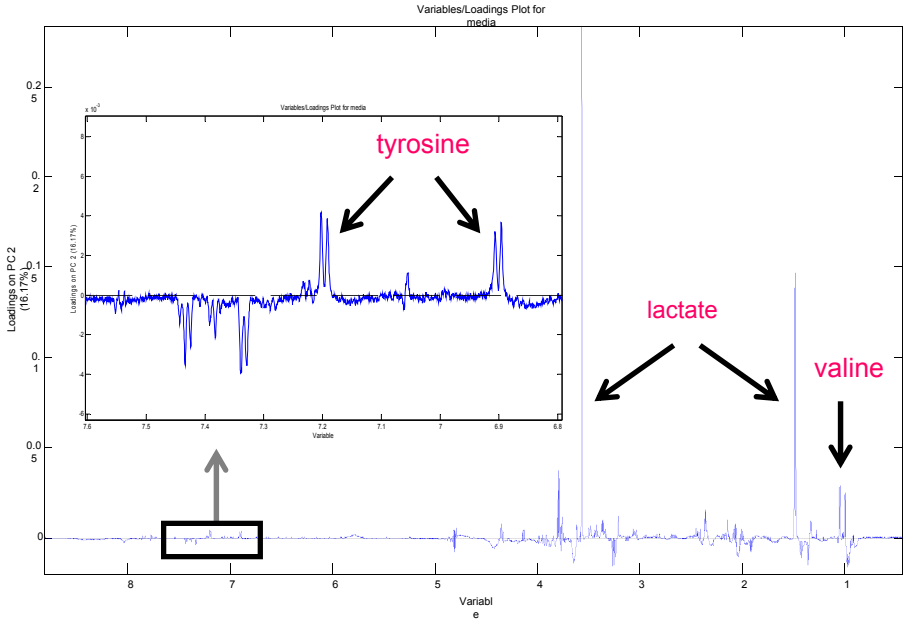
- Once more than 123 pathovars, but following revisions, now 6 pathovars - infect cruciferous plants

- X. *campestris* pv. *campestris*
  - *Black rot broccoli*
- X. *campestris* pv. *raphani*
  - *Leaf spot rocket*
- X. *campestris* pv. *incanae*
  - *bacterial blight stock*

Method

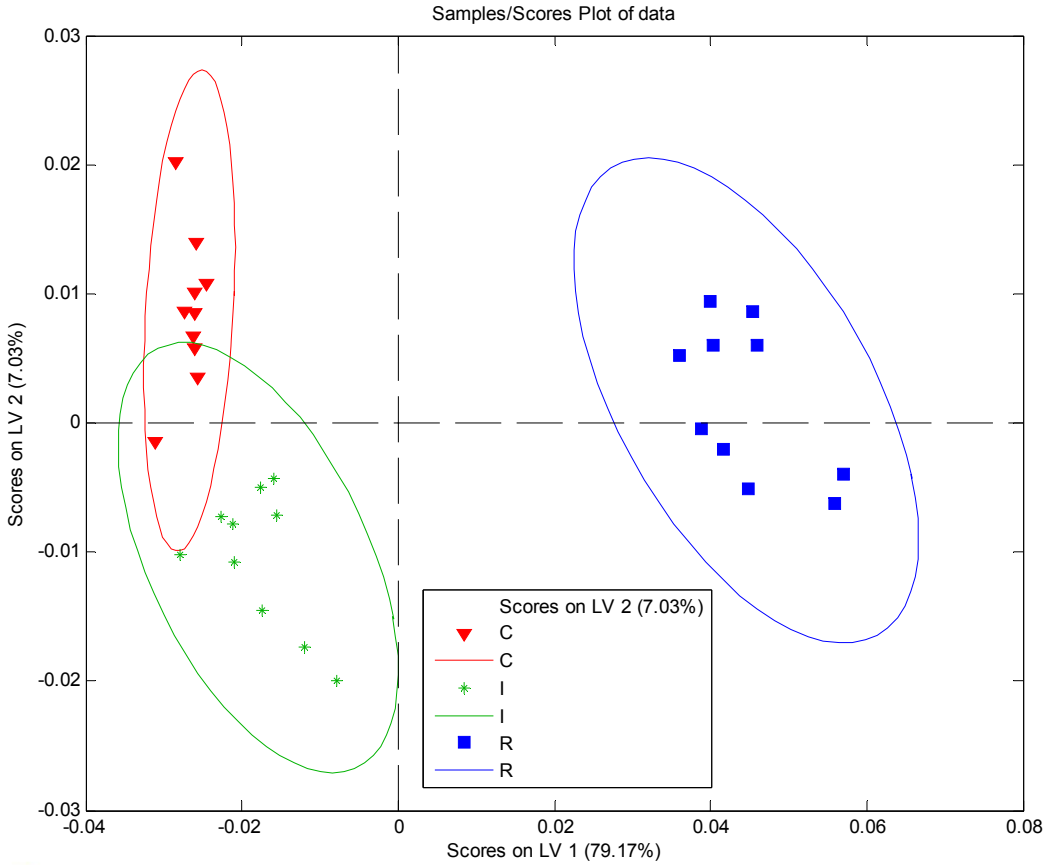
- Bacterial cell pellet and supernatant
- Inoculated plants sampled at 7 and 14 days
- NMR and LCMS analysis

NMR loading plots



NMR (supernatant) PCA loading plots showing sugars, lactate and amino acids peaks

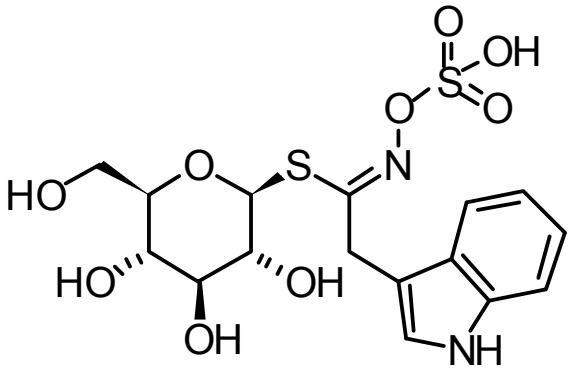
Separation of pathovars



PCA plot demonstrates good separation between pathovars

Xcr is a non-vascular pathogen, whilst *Xci* and *Xcc* are vascular pathogens and thus more closely related.

Glucobrassicin



Glucobrassicin is a highly active egg-laying stimulant of cabbage white butterflies
Also has anti-cancer properties

Metabolomics

- Total of 532 samples prepared to date
- Next steps
  - Test further on multiple isolates of pathovars
    - 8 each of A, A*, Aw
    - 6 x *malv*acearum
    - 2 x E
- Interpret results to identify suitable metabolite biomarker(s) for differentiation

Honours project (2010) – see poster!

Differentiation of *X. campestris* pathovars using metabolomic profiling – Simone Vassiliadis

(Supervisors: Simone Rochfort, Jo Luck and Kim Plummer)

1. Three pathovars of *X. campestris* could be clearly separated based on the whole metabolomic profile of bacterial cells and inoculated brassica hosts
2. Further separation was also observed between isolates of the same pathovar suggesting "sub-pathovar" discrimination (too sensitive?)
3. Pathovar separation was associated with their mode of pathogenicity (vascular versus non-vascular)

Further work

- Unique biomarkers need to be isolated for further diagnostic development
- More pathovar isolates need to be analysed

Acknowledgements

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- Murdoch Uni (SABC) : David Berryman, Celia Smuts, Mike Jones, Mehmet Cakir

THANKYOU !